Attachment A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:Confirmation No. 7027
Serial No. 10/572,920	: Group Art Unit 1636
Hiroo Iwata et al.	: Attorney Docket No. 2006_0408A
Filed : May 5, 2006	: Examiner KETTER, JAMES S

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir:

I, Hiroo IWATA hereby declare that:

I was born in Wakayama prefecture, Japan, in 1949;

I am a citizen of Japan and a resident of 1-5-8-203, Wakayamadai Shimamoto-cho Mishima-gun, Osaka 618-0024 JAPAN;

I graduated from Department of polymer chemistry, Faculty of engineering, Kyoto University, Japan in 1973.

I received my doctor degree on the study of "Synthesis of Reactive Polymers and Study of Their Polymer-Polymer Reactions" at Kyoto University, Kyoto, Japan, in 1979;

I have worked as a professor of Kyoto University in Japan from 1999 until now and have engaged in a study on Biomedical Engineering;

I am one of the inventors for this application;

I have many reports relating to electroporation using an electrode with a cationic surface. The reports are as follows:

1: Yamauchi F, Okada M, Kato K, Jakt LM, Iwata H., Array-based functional screening for genes that regulate vascular endothelial differentiation of Flk1-positive progenitors derived from embryonic stem cells. Biochim Biophys Acta. 2007 Aug;1770(8):1085-97.

2: Yamauchi F, Koyamatsu Y, Kato K, Iwata H., Layer-by-layer assembly of cationic lipid and plasmid DNA onto gold surface for stent-assisted gene transfer. Biomaterials. 2006 Jun;27(18):3497-504.

3: Yamauchi F, Kato K, Iwata H., Layer-by-layer assembly of poly(ethyleneimine) and plasmid DNA onto transparent indium-tin oxide electrodes for temporally and spatially specific gene transfer. Langmuir. 2005 Aug 30;21(18):8360-7.

4: Yamauchi F, Kato K, Iwata H., Spatially and temporally controlled gene transfer by electroporation into adherent cells on plasmid DNA-loaded electrodes. Nucleic Acids Res. 2004 Dec 21;32(22):e187.

5: Yamauchi F, Kato K, Iwata H., Micropatterned, self-assembled monolayers for fabrication of transfected cell microarrays. Biochim Biophys Acta. 2004 Jun 11;1672(3):138-47. Erratum in: Biochim Biophys Acta. 2004 Sep 6;1674(1):109-10.

6: Fujimoto H, Kato K, Iwata H., Prolonged durability of electroporation microarrays as a result of addition of saccharides to nucleic acids. Anal Bioanal Chem. 2009 Jan;393(2):607-14.

7: Fujimoto H, Kato K, Iwata H., Electroporation microarray for parallel transfer of small interfering RNA into mammalian cells. Anal Bioanal Chem. 2008 Dec;392(7-8):1309-16.

8: Fujimoto H, Kato K, Iwata H., Use of microarrays in transfection of mammalian cells with dicer-digested small interfering RNAs. Anal Biochem. 2008 Mar 15;374(2):417-22.

9: Inoue Y, Fujimoto H, Ogino T, Iwata H., Site-specific gene transfer with high efficiency onto a carbon nanotube-loaded electrode. J R Soc Interface. 2008 Aug 6;5(25):909-18.

10: Fujimoto H, Yoshizako S, Kato K, Iwata H., Fabrication of cell-based arrays using micropatterned alkanethiol monolayers for the parallel silencing of specific genes by small interfering RNA. Bioconjug Chem. 2006 Nov-Dec;17(6):1404-10.

11. Koda S, Inoue Y, Iwata H., Gene transfection into adherent cells using electroporation on a dendrimer-modified gold electrode. Langmuir. 2008 Dec 2;24(23):13525-31.

12. Inoue Y, Fujimoto H, Ogino T, Iwata H. Site-specific gene transfer with high efficiency onto a carbon nanotube-loaded electrode. J R Soc Interface. 2008 Aug 6;5(25):909-18.

13: Njatawidjaja E, Iwata H., Gene delivery to cells on a miniaturized multiwell plate for high-throughput gene function analysis. Anal Bioanal Chem. 2008 Oct;392(3):405-8.

The experiments given below were conducted under my supervision.

Experiment

(1) Purpose

The experiment was designed to compare the gene expression of living cells in the electroporation wherein an electric pulse was applied to cells adhered onto the surface of nucleic acid-loaded electrode with the gene expression of living cells in the electroporation wherein an electric pulse

was applied to cells suspended in a medium using nucleic acid-loaded electrode.

(2)Method

Example A

A glass electrode on which a 1nm-thick Cr monolayer and a 49nm-thick Au monolayer are formed in this order was used. The electrode was immersed in 1mM 1-mercaptoundecanoic acid, which has a carboxyl group at the terminal, thereby a self-assembled monolayer was formed on the Au monolayer. The resultant main electrode was washed and immersed in a phosphate buffered saline(pH=7.4) containing polyethyleneimine having an average molecular weight of 25000 at a concentration of 0.2 wt% for 1 hour, thereby a polyethyleneimine monolayer was formed on the self-assembled monolayer. Then, a 0.5 mm-thick silicone coat was formed on the rim of the polyethyleneimine monolayer. Thus, the main electrode was obtained.

100 µl of an aqueous solution containing 10µg/ml fibronectin and 10µg/ml plasmid DNA was deposited on the main electrode and incubated at 37 ℃ for 1 hour, thereby the plasmid DNA was adsorbed on the surface of the main electrode. The plasmid DNA is pDsRed-c1 capable of expressing fluorescent protein DsRed.

A cell suspension was prepared by suspending HEK293 cells in a serum-free OptiMem medium at a concentration of $40x10^4$

cells/ml. 100 µl of the cell suspension was deposited on the DNA-loaded main electrode.

A facing electrode, which is a glass electrode on which a 1nm-thick Cr monolayer and a 1nm-thick Au monolayer are deposited in this order, was immobilized at an opposed position of the main electrode with 0.5nm gap.

The cells were incubated at 37 ℃ under 5% CO_2 atmosphere for 4-5 hours to adhere to the surface of the DNA-loaded main electrode.

The main electrode was set as cathode (-) and the facing electrode was set as anode (+). They were connected to a high voltage pulse generation device (Xcell system manufactured by Bio-Rad Laboratories) and an electric pulse was applied under the conditions of the field strength of 14V/cm, the pulse duration of 10 msec and the number of pulse application of 1 to carry out electroporation.

Immediately, the facing electrode was removed and the main electrode was shifted to a maintenance medium containing 10% serum and was left at rest at 37 ℃ for 3 days.

Three glass electrodes were employed for electroporation and gene tranfection efficiency was obtained by averaging efficiency values for these electrodes.

<u>Comparative Example A</u>

A DNA-loaded main electrode without application of a cell suspension was obtained in a similar manner of Example A.

A facing electrode, which is a glass electrode on which a 1nm-thick Cr monolayer and a 1nm-thick Au monolayer are formed, was immobilized at an opposed position of the main electrode with 0.5nm gap.

100 μl of the cell suspension (HEK293 cells in a serum-free OptiMem medium at a concentration of $40x10^4$ cells/ml) was applied between the electrodes.

The main electrode was set as cathode (-) and the facing electrode was set as anode (+). They were connected to a high voltage pulse generation device (X cell system manufactured by Bio-Rad Laboratories) and an electric pulse was applied under the conditions of the field strength of 14V/cm, the pulse duration of 10 msec and the number of pulse application of 1 to carry out electroporation.

The electrode pair was left at rest at 37 ℃ for 4-5 hour in order to adhere the cells to the surface of the main electrode. Then, the facing electrode was removed, and the medium was removed and a maintenance medium containing 10% serum was applied, and the cells on the electrode were left at rest at 37 ℃ for 3 days.

(4) Result

The above mentioned experiments (Example A and Comparative Example A) were carried out 3 times, respectively. The cells adhered to the main electrode were collected for every

electroporation and the mixture of them were subjected to the test mentioned below.

The living cell number was counted using a hemocytometer.

The fluorescent intensity of the cells was measured using a flow cytometry. The fluorescent intensity indicates the average of fluorescent intensity of one living cell. Therefore, the product of fluorescent intensity and living cell number indicates the overall judgment of cell survival rate and living cell gene expression.

The results are shown in Table 1 below.

Table 1

	Living cell number/electrode	Product of fluorescent intensity and living cell number
Example A	25867	4.42
Comparative Example A	4667	1.41

(5) Discussion

An electric pulse was applied to cells adhered to the DNA-loaded electrode in Example A, while, an electric pulse was applied to cells suspended in a medium in Comparative Example A.

The gene expression of living cells of Example A was 4-times higher than that of Comparative Example A.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18U.S.C.1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 28 April, 2009

Hiroo Iwata

Hiroo IWATA